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A Research Note

Chiral Liquid Chromatography for Resolving Malic Acid Enantiomers in Adulterated Apple Juice

L. W. DONER and P. J. CAVENDER

ABSTRACT

Reversed-phase HPLC with an aqueous mobile phase containing the chiral ligand-exchanger Cu^{II}/ (N,N-dimethyl-L-valine)$_2$ resolved the enantiomeric α-hydroxy acids, D- and L-malate. Post-column detection with acidic Fe^{III} resulted in specific detection of α-hydroxy acids, so filtered apple juice gave a simple profile. D-Malic acid in apple juices suspected of being adulterated with synthetic DL-malic acid is presently determined from the difference between DL-malic acid (HPLC assay) and L-malic acid (L-malate dehydrogenase assay). The potential of the chiral HPLC method relative to the indirect method was evaluated and additional possibilities for direct and more sensitive determination of D-malate were suggested.

INTRODUCTION

CONSIDERABLE EFFORT has been directed to examining apple juices for adulteration. Pure apple juice contains from 150 to 910 mg/100g of L-malic acid (Mattick and Moyer, 1983), and the adulterative addition of synthetic DL-malic acid is indicated by the presence of the D-enantiomer. Since the cost of pure L-enantiomer precludes its addition, efforts have been directed to detecting D-malic acid. A direct and sensitive method for determining D-malic acid in suspect apple juices is needed.

L-Malic acid can be accurately determined by L-malate dehydrogenase catalyzed oxidation followed by colorimetric determination of the reduced co-factor NADH (Mollering, 1974). In conjunction with an HPLC method for measurement of total DL-malate, the presence of the D-enantiomer is indirectly determined by difference (Evans et al., 1983). An inter-laboratory study has shown that the precision of this procedure was quite good (Zyren and Elkins, 1985). Addition of synthetic DL-malate to the 20% level, however, was required before apple juice could be classified as adulterated at a 95% confidence level.

Benecke (1984) reported that enantiomeric α-hydroxy acids, including D- and L-malate, are efficiently resolved by ligand-exchange HPLC. A reversed-phase column was used along with a chiral mobile phase consisting of a complex of Cu^{II} and N,N-dimethyl-L-valine. α-Hydroxy acid complexes with Fe^{III} were selectively detected at 436 nm to concentrations as low as 4 μg.

The purpose of this study was to test the application of chiral HPLC to direct determination of D-malate in adulterated apple juices. As a result of rapidly advancing chiral HPLC and HPTLC technology and the occurrence of a natural source for D-malic enzyme, other possibilities were also suggested.

MATERIALS & METHODS

Reagents

The D- and L-enantiomers of valine and malic acid were purchased from Sigma Chemical Co. (St. Louis, MO). A simulated apple juice was prepared by dissolving 5.7g fructose, 3.1g glucose, 1.1g sucrose and 0.5g DL-malic acid in 100 mL water. Authentic juices were prepared from several varieties of apples purchased locally, and filtered (nylon, 0.22μ) prior to HPLC injection.

N,N-Dimethyl-D- and L-valine (D-DMV and L-DMV) were prepared by reductive methylation, modifying an existing procedure (Bowman and Stroud, (1950). D- or L-Valine (12.5g) was dissolved in a mixture of 260 mL water and 40.2 mL of 36% formaldehyde (aqueous). Palladium (10%) on charcoal (10g, Aldrich Chemical Co., Milwaukee, WI) was added and the mixture shaken for 2 hr under an initial 50 psi hydrogen (with extreme care!). The reaction mixture was filtered through Celite, the residue washed with 200 mL ethanol, and the combined filtrates evaporated to about 30 mL on a rotary evaporator. To this 30 mL was added 100 mL water, and the solution was reconcentrated to 30 mL. The addition of water and reconcentration of 30 mL was repeated three times, and after the third time the solution was evaporated to dryness. These repeated additions of water and concentrations were required to convert paraformaldehyde (produced by concentrating the excess formaldehyde in the original reaction mixture) into formaldehyde, which was removable by rotary evaporation. The white solid mass resulting from evaporation to dryness was dissolved in 100 mL ethanol under reflux. After storing overnight at 5°C, the crystalline needles of D-DMV and L-DMV, respectively, were filtered and recrystallized from ethanol [yield, 8.48g (55%); mp 154°C, (Bowman and Stroud, 1950) mp 154°C].

HPLC procedures

An aqueous mobile phase containing either D-DMV or L-DMV (8 mM) and cupric acetate (4 mM) was delivered at 1.5 mL/min with a Perkin-Elmer Series 3 liquid chromatograph equipped with a C-8 HPLC column (4.6 × 250 mM, Alltech Assoc., Deerfield, IL)), a 20 μL injection loop (Rheodyne) and a variable wavelength detector set at 436 nm. The post-column reagent (2.5 mM $FeCl_3$, adjusted to pH 1.5 with 70% $HC10_4$), was delivered at 0.3 mL/min (ISCO model 314 metering pump, Omaha, NE) to a mixing T, where it merged with the column eluent. Peaks representing D- and L-malate were monitored with a Hewlett-Package 3990A integrator-recorder, and elution order was determined by injecting each pure enantiomer.

RESULTS & DISCUSSION

WHEN THE Cu^{II}(L-DMV)$_2$ complex was used as the mobile phase reagent, D-malic acid eluted from the C–8 column before L-malic acid, with retention times of 5.12 and 6.35 min. (Fig. 1A). Elution order was reversed when D-DMV was used as the mobile phase reagent (Fig 1B). Pure juice from several varieties of apples were tested, and all gave chromatograms similar to that shown in Fig. 1C. The unidentified peak present in all pure apple juices tested did not interfere with D-malate, obviating the need to use the more expensive D-valine in the preparation of mobile phase D-DMV.

A simulated apple juice containing synthetic D,L-malic acid was prepared for use as an adulterant. A chromatogram of pure apple juice adulterated to the 25% level with the simulated juice is shown in Fig. 1D. The first peak represents D-malate from simulated juice, the second peak is the unidentified peak present in pure apple juices (Fig. 1C) and the third peak contains L-malate from both adulterant and the pure juice component of the mixture. On occasion, although not reproducibly,

Authors Doner and Cavender are with the USDA-ARS, Eastern Regional Research Center, 600 East Mermaid Lane, Philadelphia, PA 19118.

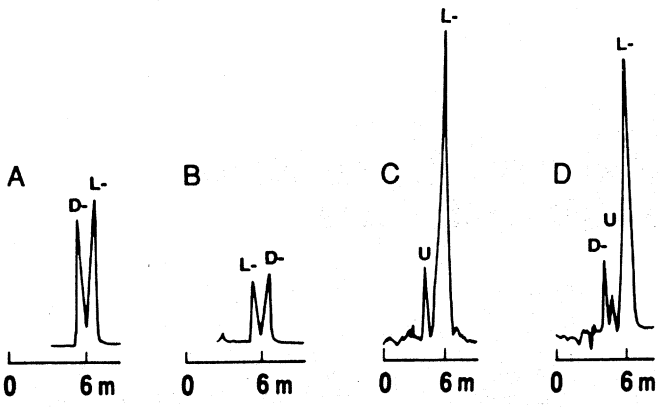

Fig. 1—High-performance liquid chromatograms of A, 70 μg each of D- and L-malic acid; B, 35 μg each of D- and L-malic acid; C, pure Granny Smith apple juice; and D, apple juice adulterated to 25% level with simulated juice. N,N-Dimethyl-L-valine was used as the chiral mobile phase reagent in A, C, and D, while N,N-dimethyl-D-Valine was used in B.

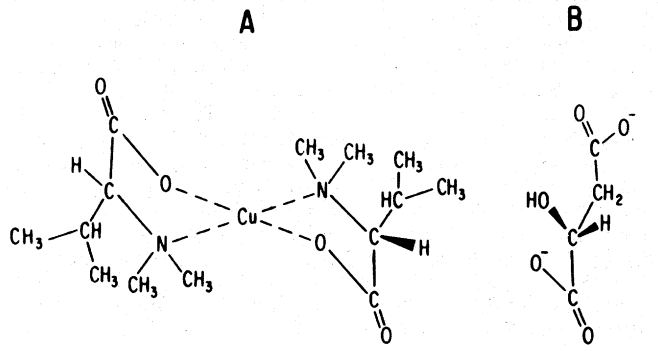

Fig. 2—A, Structure of the chiral mobile phase reagent Cu^{II} (N,N-dimethyl-L-valine)$_2$; B, D-malic acid.

the simulated juice in admixture with pure apple juice at levels as low as 10% has been detected.

The structures of the mobile phase Cu^{II}/ (L-DMV)$_2$ complex and of D-malic acid are shown in Fig. 2. Separable diastereomeric complexes form when D- and L-malate displace one of the L-DMV moieties from Cu^{II} ion. Possible mechanisms for such ligand-exchange enantiomeric separations have been discussed by Armstrong (1987). A related HPLC method for improving upon the current indirect procedure (Evans et al., 1983) for detection of D-malate in suspect apple juices is available. In a study unrelated to the adulteration problem, Horikawa et al. (1986) described the resolution of malic acid enantiomers using underivatized valine and Cu^{II} ion in the mobile phase. This procedure avoided the tedious synthesis of L-DMV, but detection by post-column reaction with Fe^{III} was required.

Direct enzymatic assay for D-malate became a possibility with the report of Knichel and Radler (1982), who isolated D-malic enzyme from *Pseudomonas fluorescens*. This enzyme oxidatively decarboxylates D-malate to pyruvate and carbon dioxide and allows very sensitive and specific determination of D-malate in fruit juice. D-malic enzyme is not commercially available, so its application to the adulteration problem has been hindered. Additional possibilities are chiral HPTLC plates (Brinkman and Kamminga, 1985) and HPLC columns to which chiral phases are covalently bound. These phases have recently become commercially available and effectively resolve α-amino acid enantiomers. It is likely that conditions could be developed for resolving enantiomeric α-hydroxy acids by use of these new phases.

A direct and more sensitive method for determining D-malic acid in suspect apple juices is needed. The procedure described here successfully resolved malic acid enantiomers, with apple juice giving a simple HPLC profile. The synthesis (employing catalytic hydrogenation) of mobile phase L-DMV and a specific post-column reaction for malic acid were required. These procedures are hazardous and tedious, respectively and desired sensitivity was not achieved. We regard the D-malic enzyme the method referred to above as the best possibility for routine D-malate determination. Until the enzyme is more widely available the continued use of the indirect method for D-malate, based on combined enzyme/HPLC analysis is suggested.

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